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Alterations in Macrophage Functions By Environmental Chemicals

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The establishment of infectious diseases is rarely entirely attributed to a single entity, but instead is the result of a primary stress and one or more secondary factors that interfere with homeostasis and the ability of the host to cope with the primary etiologic assault. Any environmental chemical that can suppress the normal functioning of the host's body defenses would be expected to increase the risk of the host to such diseases. Within the lung, the alveolar macrophages are the crucial elements responsible for defending the body against such airborne viable agents. The effects of inhaled gases and particulates on these defense cells are a major concern of the environmental health scientist since such chemicals have the capability of adversely affecting the integrity and functioning of these pulmonary defense cells. The objective of this report is to provide an overview that will improve our understanding of how a variety of environmental chemicals can alter the biochemical, physiological and immunological functioning of these cells.

The resident population of alveolar macrophages within the normal lung is crucial in the defense of the lung against environmental hazards. The responsibility for host defense is diverse and varied, including such important activities as detoxifying and/or removing inhaled particles; maintaining sterility against inhaled microorganisms; interacting with lymphoid cells in immunity; and removing damaged or dying cells. To perform these duties adequately, the cells must maintain an integrated membrane, full mobility, phagocytic activity and have a functioning enzyme system. Any alteration in the functioning of any of these individual components would be expected to increase the risk of the host to disease.

Numerous texts and monographs have been written that provide valuable references to the complexity of the integrated pulmonary host defense system and the relationship of the alveolar macrophage to the health of the host (*1–5*). These documents testify to the recent advancements in our understanding of the biochemical, physiological and immunological mechanism of action of these cells.

Instead of dealing with the role of the normal macrophage in host defense, the theme of this review will be to discuss those environmental assaults that impair alveolar macrophage's activities. This paper is not intended to review completely all available data on every chemical tested nor will it provide dose-response data. Instead, it is an attempt to provide the reader with a selective, but representative, overview of major advances in the field. An extensive bibliography is included which will guide the reader to additional information on the subject. The objective of this review is to assess the current approaches and techniques for studying the effects of environmental chemicals on alveolar macrophages by illustrating the effects and chemical compounds that have been studied and relating the observed changes to the potential health risk of the host. It is hoped that this paper will stimulate the reader's curiosity and provide ideas for future experimentation which will aid in improving our understanding of the relationship between altered host defenses and the pathogenesis of pulmonary disease.

Since the respiratory tract is one of the most common routes of entry for numerous agents, the alveolar macrophage, as a first line of defense, may be adversely affected either through direct contact and/or through the interaction intracellularly with these airborne chemicals that have successfully eluted the absorptive and filtering action of the upper respiratory passage.

Within the lung, macrophages may be found either in the interstitial connective tissue of the alveolar wall, fixed to the alveolar wall, or free in the lumen of the alveolus (*2,4*). In the main, it is impossible to study the actual numbers and specific functioning of these cells under *in vivo* conditions. For this reason, it is necessary to remove the macrophages from the lungs by using the pulmonary lavage technique to obtain relatively large numbers of these free cells without artificial stimulation. There are still many macrophages remaining in the lung after lavage but most of the cells obtained are actively phagocytic and are thought to be representative of the

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lung's defense cells. Such studies were first initiated by La Belle and Brieger (*6*) to study the elimination of dust from the lung. Using this technique, macrophages have been harvested from a variety of species: swine (*7*), rats (*8,9*), cats (*10*), dogs (*11*), rabbits (*12*), hamsters (*13*), guinea pigs (*14*), monkeys (*15–17*), mice (*18,19*) and man (*20–25*). In these experiments, the basic techniques of lavage, lavage fluid, number of washes, amount of fluid instilled, age, sex, weight, etc., often varied. It should be kept in mind that care should be taken when attempting to compare different studies in that these variables can make a difference in the recovery of these cells.

The parameters of number, stability, viability, morphology, function and biochemistry/metabolism have been used to appraise the conditions of the macrophages. In general, each criterion is used to assay for a specific change that may indicate the "health" of these cells. However, for understanding and assessing the effects on the macrophage, it is necessary to examine most, if not all, of these criteria since the endpoint affected may be specific for any given chemical. Of these parameters, the most useful are the measurements of functional capacity (i.e., phagocytic index, bacteriocidal capacity) and the maintenance of cellular integrity.

Alterations in Numbers

There is evidence from genetic chimera studies utilizing chromosomal and immunological markers and radiotracer deoxyribonucleic acid labeling techniques that the alveolar macrophage belongs to the mononuclear phagocyte system and that the majority of these cells is derived from bone marrow monocytes (*2,5,26*). Under normal conditions, the number of these cells is relatively constant. Such a steady state is a necessary condition for proper physiological homeostasis. Contamination of inhaled air with airborne particles or gaseous pollutants can upset this steady-state kinetics of the normal macrophage population and either act as a stimulus to recruit the macrophage migration from the lung interstitum or lung capillaries or can result in cell loss if the chemical is cytotoxic. Both of these events may cause in themselves a detrimental effect on the health of the host. For this reason, there have been numerous attempts to estimate the total alveolar macrophage population in normal and pollutant-exposed animals (*27–31*).

A compound that can alter the number of defense cells and their clearance in the lung is likely to enhance the action of the toxic material both on the lung and other sites of the body. During periods of pulmonary insults, the clearance rate of the deposited particles from the alveoli is a prime function of the resident macrophage. Data indicate a positive correlation between the amount of particles cleared and the number of these phagocytic cells in the lung (*18*). A number of inhalable substances such as carbon (*1,6,18,32*), diesel exhaust (*33*), PbO (*34*), $NiCl_2$, NiO (*35–37*), $CdCl_2$ (*38*), CO (*39*), Pb_2O_3 (*8,40*), cigarette smoke (*24,41–43*), cotton dust (*44*), quartz (*45*) and other environmental substances (*46,47*) can promote the influx of macrophages into the pulmonary racemus. Little is known about the mechanisms of action that stimulate this migration or about the fate of these immigrant cells. Particles, depending on their total deposited mass, size and chemical composition, can cause chemotaxis of new macrophages into the lung (*48*). This relationship is maintained whether the substance was administered by inhalation or by intratracheal instillation. The sources of these new cells may be from either (1) the influx of interstitial macrophages; (2) the proliferation of interstitial macrophagic precursors with subsequent migration of the progeny into the air spaces; (3) migration of blood monocytes; or (4) division of free alveolar macrophages. Evidence seems to indicate that the rapid increase in the number of macrophages may be a biphasic response, involving an early phase apparently correlated to a local cellular response and a later phase of interstitial cell proliferation that is responsible for the maintenance of the high influx of macrophages (*18,41,45*). ,Thus, the lung has the capacity to respond to potentially hazardous particles by increasing the number of cells from the interstitium reserve and/or the feeder monocytes in the blood.

Such marked accumulation of macrophages may appear to be a reasonable response to the immediate insult, but the possible consequences of this mass recruitment may also be instrumental in the development of future pulmonary disease. The ultimate fate of these phagocytes is often determined by the cytotoxic potential of the particle which is ingested. If the macrophage is not adversely affected by the ingested substance, the material will most likely be cleared from the lung via the ciliary transport mechanism (*5*). It has been estimated that from 1 to 5 million macrophages per hour leave the lung by this route (*5*). In these cases, bacteria and viral agents are usually killed or inactivated, acids are neutralized, antigenicity is altered, and particles solubilized. In some cases, such as following PuO_2 inhalation, the macrophage may die within the lung, releasing the ingested substances which are then phagocytized by other macrophages. The possibility also exists that the macrophage may be removed via some other mechanism, for example, through the epithelium and into the lymphatics to be drained off with the lymph. This idea is quite controversial. The most recent evidence for the existence of this pathway is presented in a review on the role of the lymphatics in pulmonary clearance (*49*). Particle-laden macrophages can also be observed within the septal interstitium, but whether these particles entered the tissue directly as free particles and subsequently phagocytized by the fixed macrophages or whether the particles were first phagocytized in the air spaces and then carried into the interstitium by migrating free cells is a question still unanswered. This information is vitally important for the environmental toxicologist. If inhaled substances

are able to gain access directly through the epithelial barrier, not only would the retention time be greatly increased, but there could also be an accumulation of those toxic substances resulting in a greater risk to the host of producing chronic respiratory disease.

Not all environmental chemicals that reach the lung elicit an automatic influx of reserve macrophages. Exposure to a number of agents reduces the number of free macrophages: lead sequioxide (*8*), silica (*45,50, 51*), asbestos (*50,52*), Pb_2O_3 (*8*), Sb and Cd fumes (*53*), MnO_2 (*54*), Mn_3O_4 (*55*), O_3 (*56*), chrysotile (*57–59*), amosite (*60*), CdO and $CdCl_2$ (*53,61,62*), acrolein (*63*) and $NiCl_2$ (*35*). These chemical agents are cytolytic to these defense cells. It is interesting to note that although the residential macrophages are significantly reduced in numbers immediately after the exposure, there does not appear to be any immediate new influx of reserve cells to cleanse the lung. It appears that either these substances are so toxic that the new cells are also quickly eliminated or they inhibit the movement of the reserve cells into the air spaces. Histological studies have reported that with increasing the time of exposure to O_3 and NO_2, there is evidence of an accumulation of macrophages within the proximal alveoli of the alveolar ducts (*64–67*). NO_2 appears to be able to stimulate macrophage division but does not trigger the infiltration of additional macrophages into the alveoli, as demonstrated by cell population counts (*68*).

Continuous exposure of mice to 90% oxygen causes disintegration of endothelium and Type I epithelial cells but no increase in alveolar macrophages. The authors used this information to suggest a differential response of pulmonary cells to oxygen, that is, that the macrophages were more resistant to hyperoxia (*69*).

Alterations in Viability and Cell Types

In addition to enumerating the total number of cells isolated from the lung, it is imperative that two other measurements are also made. These include (1) the percent of the isolated cells that are viable and (2) the determination of cell types. One cannot assume that all of the cells are viable, nor that a homogeneous sample of pure macrophages has been obtained. Cell viability is most often determined by the trypan blue dye exclusion test (*70*); however, other stains (*59,71*), as well as other methods, such as measuring the release of soluble cytoplasmic enzymes, i.e., lactate dehydrogenase, lysozyme secretions into the medium, have been used to estimate cell viability (*24,72*).

Chemicals may affect the cellular viability and not cause lysis. This was shown when the cytotoxicity of several trace metals, Cd^{2+}, Mn^{2+}, Ni^{2+}, Cr^{3+} and VO_3^-, were tested in an *in vitro* macrophage system (*73*). All metals except Cd^{2+} produced a significant decrease in number of cells at concentrations that also affected cell viability. However, Cd^{2+} was unique in that it decreased cell viability without causing lysis. Thus, failure to determine cell viability can lead to gross misinterpretation of the data. Human alveolar macrophages cultured with amosite asbestos showed no loss in cell viability at concentrations less than 100 μg/mL but significant cytotoxicity was observed at higher concentrations, > 100 μg/mL (*60*).

Other *in vitro* investigation have studied the cytotoxicity of "real world" fly ash particulates collected from different industrial processes including coal-fired power plants, steel foundry, and copper and aluminum smelters. The estimated concentration required to reduce the macrophage viability to 50% was greater than 950 μg/mL for all samples except the copper and aluminum dust, which required 11 and 114 μg/mL, respectively (*74*). The most toxic samples released some soluble cytotoxic substance into the medium that was responsible for the decrease in viability. When fly ash was coated with various metals and tested for its effects on macrophage viability, the results indicated that PbO-treated fly ash was most toxic, NiO- and MnO_2-treated particles had an intermediate effect, and the untreated fly ash was the least toxic (*75*). These data also show that as the particle size increases, a significantly greater concentration is required to reduce viability to the same level.

Other investigators (*34*) have reported that PbO has a low order of toxicity for the macrophage. The intratracheal instillation of moderated doses of PbO (no evidence of an inflammatory response) caused a significant increase in macrophage numbers, but the viability was relatively constant.

Exposure to CO also decreased the macrophage viability of recovered cells (*39*). This effect was noticed regardless of changes in partial pressure. With this decrease in viability there was a 5- to 6-fold increase in macrophage counts for all levels tested. The authors believe that the decreased viability was compensated for by a simultaneous increase in number of macrophages.

It is not only the macrophages that migrate into the target site during periods of pulmonary insults. A number of respiratory irritants elicit an active influx of granulocytes from the blood. The accumulation of polymorphonuclear (PMN) leukocytes in the alveoli has been reported following acute exposure to diesel exhaust (*33*), O_3 (*56,76*), NO_2 (*77*), iron oxide (*78*), cotton dust (*44,79*), cigarette smoke (*22,80,81*), HCl (*82*) and $CdCl_2$ (*38,61,62,83*). This rapid influx of PMN has been shown to be dose-related and is a useful measurement parameter for evaluation of a pulmonary inflammatory response. Although the mechanism for this influx of PMN is not known, there is evidence that the macrophages can secrete a low molecular weight chemotactic factor that preferentially attracts PMN (*22, 84*). This factor can be isolated from bronchopulmonary lavage fluid (*85*). It appears that this response is chemical-specific since inhalation of particles of carbon (*79*), barium sulfate (*79*), Mn_3O_4 (*55*), $NiCl_2$ (*35*), fly ash (*86*), chrysotile (*59*) and silica (*79*) did not recruit leukocytes at the concentrations tested.

It would seem logical that the PMN, which are now free cells in the alveoli, were transported to this area of inflammation to provide a rapid and potent defense against the causative agent and to combat infection. Unfortunately, studies to examine the efficiency of the functioning of these cells in their new environment are lacking as well as their susceptibility to the test chemicals. Once PMN accumulate in the lung, it is possible that they may actually interfere with macrophage functions (*22*), i.e., migration, chemotaxis or phagocytosis.

Examining the cell population in the lavage fluid is also a simple task for determining the health of the normal animals. An increase in PMN is a good early indicator that the animal has pre-existing respiratory problems and should not be used in the experiment.

Morphological Alterations

Morphological studies on the alveolar macrophages lavaged from the lungs of humans and animals exposed to a number of different environmental chemicals have indicated distinct changes in the structure of these cells. Tobacco smoke, in addition to filling the cytoplasm with pigmented residues of the smoke (*20,87*) also causes an increase in mean maximum diameter (*43,88, 89*). The cells are more rounded in appearance, with little or no ruffling of the cell membrane that is commonly seen in normal macrophages. There were membranous bloblike extensions that covered the entire cell (*81*). Subcellular morphology of the macrophages also exhibited some changes. The volume density of the nucleus in total cytoplasm was significantly less while the volume density of the mitochondria was slightly increased. There was no increase in number of mitochondria. The results of other measurements were (1) an increase in surface density of the mitochondrial inner membrane in total cytoplasm; (2) a decrease in the surface density of the rough endoplasmic reticulum; and (3) an increase in cytoplasmic lipids. No changes were noted in the surface densities of the smooth endoplasmic reticulum and Golgi membranes (*88,90*).

Macrophages from animals exposed to nickel dust differed from control, in that they had numerous microvilli and long protrusions from the cell surface. No difference was noted between controls and nickel-exposed rabbits on amount of mitochondria, tough and smooth endoplasmic reticulum, Golgi cisternae and ribosomes. $CdCl_2$ also caused both an increase in size of macrophages, and the appearance of numerous prominent cytoplasmic vacuoles.

Both *in vivo* and *in vitro* studies showed the effects of quartz and asbestos on the surface morphology of the alveolar macrophage (*91,92*). After ingestion of asbestos, macrophages developed large flattened pseudopodia, an increase in cytoplasmic blebbing, asbestos bodies and more extensive cytoplasmic processes (*60,93*). The cytoplasm was filled with numerous lysosomes, free and aggregated ribosomes, mitochondria, and strands of rough endoplasmic reticulum (*94*). After ingestion of quartz, macrophages showed pronounced intracytoplasmic vacuolation deterioration of the plasma membrane and assumed wild, bizarre shapes (*95*).

Scanning electron microscopy can also be used to detect morphological changes in the macrophages that precede cell death. Studying a number of trace metals, the following sequence of events in macrophage cytotoxicity was (1) retraction of normally extended pseudopodia, (2) appearance of bleblike structures on the surfaces, (3) smoothing of the plasma membrane and (4) final effacement of cell architecture (*73*).

Gaseous pollutants, such as O_3, cause ultrastructural alterations which are characteristic of a nonspecific, toxic cellular response. These effects included intracellular vacuolization, dilation of the endoplasmic reticulum and the nuclear envelope, and swelling of the mitochondria (*96*).

Alterations in Phagocytosis

The most striking functional characteristic of macrophages is their ability to distinguish foreignness and to be able to begin ingestion within minutes after particles are deposited within the lung. It is the efficiency of the phagocytic and lytic system of the alveolar macrophage that determines the sterility and health of the lung. The elimination of foreign material involves a number of integrated steps, presumably consisting of (1) chemotaxis, or the migration of the macrophage to the stimulus, (2) opsonization, the coating of the substances with complement or an immunoglobulin component, (3) the attachment of the particle to the phagocyte, (4) the engulfment of the particle and the formation of a primary phagocytic lysosome, (5) fusion with a secondary lysosome, and (6) the destruction of the ingested particle within the secondary lysosome. The complexity of this sequence of events provides numerous potential sites for perturbation by an environmental toxin (*97, 98*). Such abnormalities are manifested by compromised host defenses.

Chemicals can impair chemotaxis by destroying either the chemotactic substances or by hindering the mobility of the phagocyte to the substance. Experiments designed to estimate the rates of chemotaxis of pulmonary macrophages have been performed both by exposing the intact animal to the test chemical or by exposing normal macrophages *in vitro* and then assessing their chemotactic function. Chemotaxis can be measured *in vitro* by testing their rate of migration toward a chemotactic attractant in a Boyden-type chamber. This technique has been sufficiently standardized to assess the qualitative differences in chemotactic functioning of cells by measuring the movement of the cells through a porous filter (*99–101*). Macrophage migration can also be measured using agarose. In this technique, the macrophages and chemotactant material are placed in different wells and the linear distance between the front of the migrating cells and the margin

of the wells is determined and recorded as total migration distance (*17*). Using these techniques, one can measure both directional migration as well as spontaneous random migration. It should be noted that the rabbit alveolar macrophage responds very poorly to a chemotactic agent, and either human or guinea pig macrophages are better suited for such studies (*82*). Only a few such studies using environmental chemicals have been performed with pulmonary phagocytes. Macrophages from patients with acute smoke inhalation have marked depressed chemotaxis (*22*). A similar impairment (*22*) after *in vitro* exposure of normal macrophages to smoke has also been reported. However, other investigators (*102–104*) reported that macrophages from cigarette smokers had enhanced responsiveness to a chemotactic stimulus and an impaired responsiveness to macrophage migration factor.

Alveolar macrophages from the lungs of monkeys that had breathed O_3 demonstrated decreases in both the number of cells randomly migrating and the distance they migrated (*17*). Contrary to these effects, neither HCl nor cotton extracts impaired the chemotaxis of pulmonary macrophages (*82,105*).

In most toxicological studies designed to investigate the effect of chemicals on the phagocytic process, there is usually no attempt to distinguish between the opsonization and attachment phase and the actual ingestion of the particles by the phagocyte. The macrophages to be tested might either be those isolated from previously exposed animals or normal macrophages which had been exposed to the test substance *in vitro*. In either case, their capacity to engulf some test substance (bacteria, starch, erythrocytes, latex spheres, etc.) is compared to the control group.

One alternative approach is to inject the test substance intratracheally into the live animal and after a short *in vivo* incubation period, the macrophages with their ingested organisms can be isolated and phagocytic index enumerated. This technique allows the phagocytosis to take place within the host pulmonary environment (*56,77,106*). Within this pulmonary milieu, the host can call into action other nonspecific mechanisms of pulmonary defenses such as movement of respiratory tract fluid and specific defenses such as immune responses to aid in the phagocytosis. However, other detrimental effects of the exposure to the chemical such as edema may also be present to hinder this ingestion activity. Thus, this technique may be more natural than *in vitro* since it takes into consideration the total effects of the pollutant and the total components of the *in vivo* phagocytosis. This procedure needs to be tested and evaluated further.

The attempts to quantitate this phenomenon of phagocytosis have mainly been based on counting either the percentage of cells that can phagocytize or by enumerating the number of particles ingested by individual cells. The difficulty of this technique is determining whether the particle is free, merely attached to the surface of the cell, or actually within the cell. In attempts to remove some of this subjectivity, a number of techniques have been developed that can improve the accuracy of this assessment (*29,87,107,108*). A simple but accurate technique has been tested for determining the difference between attachment and ingestion of polystyrene latex spheres (*29*). This procedure uses xylene to dissolve the extracellular spheres, leaving the intracellular spheres to be counted.

Prior to measuring the phagocytic function of the macrophages, one must be confident that a relatively pure population of macrophages are being tested and that the ratio of macrophage to test particle is kept constant. Since many environmental agents can induce an influx of other cell types in the lung which also will be collected with pulmonary lavage, it is imperative to purify the sample.

Alveolar macrophages have been purified by the simple technique of allowing these cells to attach themselves to a plastic or glass surface and decanting the contaminating cells which will not attach. One must be careful utilizing this technique in toxicology since it may preferentially select the most active, healthiest cells and thus bias the results if attachment were affected by the test chemical. Another useful technique to obtain pure macrophages from a mixed population is by using either Ficoll-Hypaque or albumin density gradient centrifugation techniques (*109,110*). The macrophages remain at the interface while the PMN and erythrocytes are at the bottom of the tube. In measuring phagocytosis efficiency one must be aware that after heavy phagocytosis, the macrophages can enter into a refractory period. At this time, the particle uptake will be markedly suppressed for several hours (*111,112*).

Acute exposure of animals to O_3 or NO_2 (*56,77, 113–116*) produced a marked change in their phagocytic efficiency. Not only were there fewer bacteria ingested by the macrophages, but there was also a decrease in the number of cells that were phagocytic. Either extracellular or intracellular mechanisms may be responsible for this effect. A variety of possibilities exist such as these gases may (1) inactivate opsonogenic factors in the extracellular milieu, (2) alter the alveolar lining fluid, thus affecting macrophage mobility, (3) directly attack the cell membrane or (4) alter some intracellular metabolic process necessary for phagocytosis. These possibilities are not mutually exclusive and several may be responsible.

Another chemical that significantly depressed the phagocytic functioning of the macrophages was $NiCl_2$ (*35,117*). The reduction in phagocytic capability was not evident until 24 hr after the Ni exposure. *In vitro* exposure studies performed on normal alveolar macrophages have substantiated many of the effects seen *in vivo* and have provided a clue that the mechanism of action of various toxicants can vary (*118*). Phagocytosis of latex spheres was reduced by Cd^{2+}, Cr^{3+}, Mn^{2+} and Ni^{2+} (*119*). It is of interest to note that Ni^{2+} had substantial effects on phagocytosis, but had no effect on total cell lysis and only a minimal effect on

viability. On the contrary, vanadate (VO_3^-) caused a 25% reduction in macrophage numbers and a loss in viability, but no effect was observed on the phagocytic index. Other *in vitro* studies with cigarette smoke (*120–122*), NO_2 (*123*), CdO (*124*), Hg^{2+}, Cd^{2+}, Zn^{2+}, Pt^{4+}, Cu^{2+}, Ni^{2+}, and V_2O_5 (*73, 125, 126*) have also shown a reduction in the phagocytic functioning of these defense cells.

However, macrophages may also respond in a different manner. For example, macrophages exposed to nickel dust, SO_2, CH_2O had an increased phagocytic activity (*71,116,127*). Chronic exposure to $CdCl_2$ seems to increase phagocytic activity, whereas *in vitro* and acute inhalation exposure depressed this function (*83*). Thirty days of exposure to tobacco smoke caused an initial enhancement of phagocytic activity which was later reversed with more chronic exposure, and a definite inhibition of uptake then occurred (*88*). This may indicate that the effect of smoke on phagocytosis may be cumulative. Aspiration of HCl did not alter the phagocytosis of macrophages (*82*).

It is known that the size, shape, chemical composition and surface area of the environmental agent has an influence on the phagocytic functioning of the alveolar macrophages (*75,95,128,129*). Small size significantly increases the probability of deposition in the alveolar area and hence represents the greatest challenge to the defense cells. The percentage of macrophages which have phagocytized increases with decreasing particle size. Not only are the smaller particles phagocytized in larger numbers, they also provide a greater surface area for the adsorption of other potentially harmful substances (*27*). In vitro studies where trace metals were absorbed onto fly ash, indicated that PbO treated fly ash was most toxic, NiO and MnO_2 treated fly ash had an intermediate effect, and untreated fly ash was the least toxic (*75*). The data indicate that as the particle size increases, a significantly greater concentration was required to produce an equivalent effect. This is important in environmental studies since in urban air, metals are more concentrated among the smallest of the airborne particles and thus may be a significant factor in promoting pulmonary disease (*130,131*). There is also evidence that not only do the physicochemical characteristics of the particles affect the rate of phagocytosis but they apparently can also affect the endocytotic mechanisms (*49*). No differences in phagocytosis were seen between inert particles of the same size which were coated with silver, manganese, or uranium. However, particles coated with aluminum or chromium were phagocytized at a faster rate (*71*).

To interpret the results of such phagocytic studies, it is necessary to also measure viability concurrently in order to insure that the measured decrease in phagocytosis is due to functional impairment rather than a loss in viability. This can be accomplished either by mathematically accounting for the live versus dead cells or by specific staining techniques that would permit the counting of the viable cells only (*119*).

Macrophage Defense against Bacterial Agents

Because any alterations in the rate of bacterial killing may be related to impairments in pulmonary resistance to infections, there have been many studies directed at measuring the intracellular antibacterial functioning of the lung.

Some of the techniques that are used to determine phagocytic rates can also be employed to measure *in vitro* intracellular bacteriocidal killing (*106,132,133*). However, for this measurement, the test substance must be a viable microorganism. After an appropriate incubation period, the control and treated macrophages are lysed, and the number of viable organisms remaining is determined by standard plate count methods.

A somewhat simpler technique and perhaps more reflective of the total pulmonary response to the test chemical is to determine the pulmonary antibacterial activity *in vivo* (*106,114,132,134,135*). Both the treated and control animals are exposed simultaneously to an aerosol of viable microorganisms. Randomly selected animals, from both groups, are sacrificed immediately and at other time intervals. The lungs are excised, homogenized in an appropriate medium, and the number of colony forming units determined as an indicator of the bactericidal capability of the lung.

The above technique has also been expanded by using radiolabeled viable microorganisms such as *Staphylococcus aureus* (*106,136*), Klebsiella pneumoniae (*132*) and *Proteus mirabilis* (*137*). This provides a more precise measurement of the total clearance kinetics of the lung, i.e., physical and bactericidal. Using this system, the intra or extracellular localization of the intrapulmonary bacteria can also be determined histologically in the right lung, while the number of viable microorganisms are determined bacteriologically in the left lung. By measuring the total radioactivity, the actual number of deposited organisms that have been physically removed from the lung can be determined. The investigators have shown that phagocytosis of the organism by macrophages precedes and roughly parallels the actual clearance of the bacteria initially, but within 2 hr the phagocytosis reaches a plateau, while the bactericidal activity continues to increase (*99*). Comparing the normal kinetics to that of the exposed animals offers a unique measurement of the intact pulmonary antibacterial systems.

Through these types of studies and others (*134,136, 138–140*) it was found that physical removal could account for only 15 to 20% of the total bacteria cleared from the lung over a 4 hr period and that the primary *in situ* bactericidal activity was due to the macrophages. Thus, if the macrophages are capable of functioning normally, there is a rapid inactivation of the deposited microorganisms *in situ* (*5,37,135,141–144*). Growth of the bacteria within the lungs, due to the exposure to some noxious agent, would most probably be a reflec-

tion of decreased pulmonary bactericidal ability which is, in turn, caused by dysfunction of the macrophage.

The effects of exposure to NO_2 (*123, 145–147*), O_3 (*138, 141,148–150*), SO_2 (*144,151,152*), cigarette smoke (*5, 90,121,137,153–155*) silica (*156*), H_2SO_4 (*132*), Cd^{2+} (*61,124*), alumina (*124*), Ni^{2+} (*117*), Mn_2^{+} (*55,157*), ethanol (*158*), $PbCl_2$ (*159*), and high oxygen tensions (*150*) have all been investigated using model systems similar to the ones described above. Depressed bactericidal functions has been attributed to exposure to all of these substances except silica, SO_2 and O_2. Hence, exposure to numerous airborne substances can significantly alter the basic functioning of this host defense system. In many cases, these insults can be directly correlated to significant increases in susceptibility to infection. These correlations can be made on the basis of published epidemiological, occupational and laboratory experimentation. This relationship between increased concentrations of particles and gases and the increased incidence of respiratory infections will be presented later in the paper.

Macrophage Defense against Viral Agents

A few investigators have examined how exposure to environmental chemicals can decrease the ability of macrophages to combat viral respiratory infections primarily by examining the effects on interferon production. Interferon, a protein synthesized and released by macrophages when induced by viruses or polynucleotides, defends the host against viral infection by inactivating the virus (*2,83,160,161*). Any impairment in the ability of interferon production by macrophages could be a causative factor for decrease of resistance against respiratory virus infection. Interferon production by macrophages was depressed after exposure to O_3, irradiated automobile exhaust (oxidant), and NO_2 (*113,162*). Another investigator (*163*) reported that O_3 did not have any effect on the capacity of macrophages to produce interferon, but did inhibit the production of interferon by tracheal epithelium cells.

The mechanisms of resistance to infections with viruses are much less well understood than the mechanism of resistance to bacteria. However, there is a considerable amount of epidemiologic data that have shown an association between human respiratory symptoms indicative of viral infection and exposure to various airborne chemicals (*163–168*). Because of this relationship, it is important to continue to investigate how noxious agents can affect the macrophages interaction with viruses of the human respiratory tract.

Alterations in Biochemical Activities

Within minutes after phagocytosis, the lysosomes move through the cytoplasm, attach themselves to the phagosome and discharge their proteolytic enzymes (hydrolases) to digest or detoxify the captive substance. In order for this to be effective, the cell must have an adequate amount of lysosomal enzymes and there must be an orderly fusion between the phagosome and the lysosome (*5*).

Normal alveolar macrophages are rich in lysosomal enzymes, some of which are known to be involved in intracellular and/or extracellular processing of foreign materials (*5,57,169,170*). Enzymes which have been identified include acid phosphatase, acid ribonuclease, β-galactosidase, β-glucuronidase, cytochrome oxidase, lipase, lysozyme, proteases, and others (*2,4,5,170*). Because this system is crucial in the functional response of the macrophage, any perturbation of the metabolic or enzymatic mechanisms of these cells may have important consequences on the ability of the lung to defend itself against disease.

The extracellular release of such lysosomal enzymes may occur either as a result of direct cytotoxic damage and leakage of intracellular contents or they may be selectively released without any cell injury. Such selective release has been observed by electron microscopy as well as by assay of the released hydrolases in the medium (*5*). Macrophages under certain conditions secrete a number of other biologically active substances including interferon, collagenase, prostaglandins, angiogenesis factor, elastase, plasminogen activator, granulopoietins, as well as other agents that can influence tumor growth and fibroblast proliferation (*24,41,57, 160,171*). Some of these substances are secreted by macrophages but are not stored by the cells and hence cannot be detected in cell lysates (*172*).

Measurement of the intracellular or extracellular levels of such substances have been used to estimate the damage caused by the action of numerous test chemicals. *In vitro* and *in vivo* studies have demonstrated that short-term exposure to compounds of Cd^{2+}, VO_3^{-}, Ni^{2+}, Mn^{2+} and Cr^{3+} causes a reduction in specific enzyme activity such as lysozyme, acid phosphatase, etc. in macrophages (*73,125,173*). In most cases, this reduction paralleled a similar decrease in cellular viability. This was also the case when macrophages were exposed to fly ash coated with either PbO or NiO. There was a decrease in lactate dehydrogenase that followed the loss in viability (*75*). There was no change in β-glucuronidase and only a moderate decrease in acid phosphatase activity. In addition, Cd^{2+} decreases glucose metabolism and adversely affects the respiration of pulmonary macrophages by uncoupling oxidative phosphorylation and inhibiting mitochondrial oxygen uptake (*174*). Cd^{2+} and Hg^{2+} also interact with a large number of enzymes through sulfhydryl groups, resulting in the destabilization of lysosomal membranes and the subsequent release of lysosomal hydrolases (*175*). $BeSO_4$ and BeO also induce the cellular release of acid phosphatase and β-*N*-acetylglucosamidase (*176*). Pb particles were found to accumulate in the mitochondria of the macrophage which are known to be adversely affected by even small concentrations of Pb (*177*). Pb injuries the macrophages

by causing their lysosomal enzymes to leak out from the lysosomal membranes (*178*).

Particles of silica and asbestos have long been known to stimulate the release of lysosomal enzymes from phagocytic cells (*179–182*) which in turn elicits major pathological conditions such as inflammation, tissue destruction and fibrosis in the lung (*183*). In the process of the ingestion of the particles, phagosomes are formed from the invaginated cell membranes. Within hours, the phagosomes rupture, and their enzymes discharge into the cell's cytoplasm. This release of these lysosomal enzymes results in the rapid death of the macrophage and the release of its contents, including the previously ingested silica. This induces the influx of additional macrophages into this area and the cycle repeats itself. This cytotoxic effect is a necessary step in the production of the silicotic fibrosis. There is a good correlation between such toxicity and fibrogenicity for a variety of mineral dusts (*184*).

There are a number of substances that after ingestion remain within the phagosome without altering the phagosomal membrane. Dusts like carbonundum and diamond fail to show toxicity, even though they are sharp (*185*). It is thought that substances such as silica and asbestos interact differently with the cell membrane (*186*). Silica causes cell death by disrupting the phospholipid components of membranes which form hydrogen bond complexes with phenolic hydroxyl groups of silicic acid. Asbestos disrupts membranes in a nonlytic fashion by the interaction of its magnesium groups with membrane glycoproteins. Silica does not release acid hydrolases from macrophages without cell death, while small amounts of asbestos fibers induces the selective release of lysosomal enzymes (*187*).

Gaseous pollutants such as O_3, NO_2 and SO_2 caused marked alterations in enzyme activity. Sulfur dioxide increased both lysozome and acid phosphatase levels. The oxidant gases altered significantly the activity levels of lysozome, β-glucuronidase and acid phosphatase (*76,110,170,188—190*). However O_3 does not alter the range of oxygen consumption or the activity of glucose-6-phosphatase dehydrogenase (*191*). Acute exposure to low concentrations of O_3 causes decreased glucose utilization, cytochrome oxidase activity, and adenosine triphosphate (ATP) generation. No effect was observed in pyruvate kinase and phosphofructokinase activity. As the exposure time increased, the ATP and glucose utilization returned to normal (*41*). *In vitro* exposure of baboon alveolar macrophages to SO_2 causes a significant depletion of ATP, while the ATPase activity is significantly elevated (*190*). Viability and phagocytic activity are not adversely affected.

The result of action of the oxidant gases may be either on the plasma and/or lysosomal membrane (*192*). Any toxicant that affected integrity of these membranes would be expected to result in a leakage of the material extracellularly. This concept has been confirmed when it was shown that the reduction in intracellular lysosomal enzyme activity coincides with the release of the enzyme into the medium (*193*). In these studies, the sum of the intracellular plus extracellular enzyme activity did not equal the total activity, indicating that the pollutant itself can inactivate the hydrolytic enzyme as well as alter the cell membrane. It has been shown by several investigators that lysozyme is very susceptible and reacts readily to O_3 (*194*).

Cigarette smoke induces a wide range of biochemical alterations, some of which have been observed in human macrophages *(195)*. The increase in secretion of lysozyme in macrophages from both human and rodents exposed to smoke has been reported (*24,120,195–198*). This increase is associated with the production of new lysosomes and not just an increase in enzyme content within the original lysosomes (*89*). Macrophages exposed to cigarette smoke also contain less succinate dehydrogenase, cytochrome oxidase, acid phosphatase, NADH diaphorase, nonspecific esterase and have impaired ability for protein synthesis (*5,120,198,199*). Not only do smokers have greater number of macrophages, but these cells also produce greater quantities of elastase (*25,41,43,57,200,201*). The secretion of such enzymes may lead to subsequent tissue damage and connective tissue degradation. Human macrophages from smokers also release increased amounts of O_2 (superoxide anion) (*128*). Such stimulation may be a significant factor in the pathogenesis of emphysema. Cigarette smoke produces approximately a 2-fold increase in oxygen consumption and hydrogen peroxide release in response to phagocytosis (*90*). Studies have also shown that macrophages from smokers have a significantly higher aryl hydrocarbon hydroxylase (AHH) activity than similar cells from nonsmokers (*202,203*). This is important, since this enzyme system can metabolize polycyclic aromatic hydrocarbons to more active carcinogens.

It should be noted that accompanying the process of phagocytosis by macrophages there is an increased oxygen consumption, H_2O_2 and superoxide production, and simulated hexose monophosphate shunt activity (*20,204*). Such respiratory burst during phagocytosis and the accompanying production of oxygen radicals appears to be important in bacteriocidal activity (*205–208*). Overproduction of oxidants might also contribute to the inflammatory damage of emphysema. The light emitted from the oxidizing reactions (chemiluminescence) has been used as a sensitive assay for detection of oxidant production in alveolar macrophages (*209*). Air pollutant dusts as well as a number of other chemically defined particles have been examined for their activating effect on oxidant production (O_2^- and H_2O_2) in alveolar macrophages. All particles tested were found to stimulate the macrophage in a dose-dependent manner to different maximal levels of oxidant production. Three types of amphibole asbestos (anthophyllite, amosite, and crocidolite) were the most active of all agents tested. Serpentine forms were about

one-fourth as active. Silica, metal oxide-coated fly ash (PbO, NiO and MnO_2), and polymethyl methacrylate beads had intermediate activity. The lowest activity was seen with exposure to uncoated fly ash, glass fiber, polybead carboxylate microspheres, fugitive dust, glass and latex beads.

These defense cells, either because of cell injury, cell death or through selective exocytosis will release into the lung a number of biologically active enzymes, i.e., elastase, collagenase. The ability of macrophages to be stimulated independently by different stimuli to release these substances may be an advantage in resisting infection during pollutant exposure but there may also be serious consequences when the oxidant and protease secretions reach levels which could contribute to chronic lung damage (*24,41,210*). The amount of proteolytic enzymes released would increase with increasing numbers of macrophages, the cytotoxic potential of the test substance and the influx of other cells rich in similar hydrolytic substances, i.e., PMN (*200*).

Influence of Surrounding Milieu

In the lung, the macrophages are constantly bathed by an acellular lining fluid that consists of proteins, lipids and carbohydrates. This acellular fluid (surfactant) also lines the epithelial cells (Type I and II) and has as its primary function reduction of surface tension forces in the alveoli to prevent alveolar collapse at low lung volumes. Changes in surfactant have been widely shown under various pathological and experimental conditions. This lining fluid may also play an important role in the defense of the lung through its interaction with the pulmonary macrophage.

This lining material may enhance the phagocytic function of alveolar macrophages (*5,134,211*), prevent autolysis of the macrophages (*30,134,211*), enhance macrophage migration (*17,212*), improve bactericidal activity (*22,213,214*) and increase macrophage adherence to glass (*82,215*). It is reasonable to assume that certain inhaled noxious chemicals would interact with this lining fluid, altering the above macrophage functions, thus making the lung more vulnerable to infectious agents.

Both *in vivo* and *in vitro* exposure to low levels of O_3 have been shown to inhibit or destroy some factor(s) in the alveolar lining material that promotes or maintains the stability of the rabbit macrophage in suspension (*30,34,141,211*). The data indicate that some of the deleterious effects of O_3 on lung cells may be mediated through the lung fluid. Alveolar macrophages from O_3-exposed animals could also be protected partially if they were transferred to lining fluid from normal animals. It has also been shown that this material may serve as an oxidant sump to neutralize pollutant gases (*5,216*), although this sump has a limited capacity since quantitatively most of the lipids are saturated (*5,217*). Other environmental factors which have been shown to alter the lining material, resulting in adverse effects on other normal macrophage functions, include the aspiration of hydrochloride acid and thermal burns (*17,22, 82*).

The Bottom Line

In general, the consequences of any toxic response depend upon the particular cell or organ affected, the severity of the damage and the capability of the impaired cells or tissue to recover from the assault. Do such small decrements in the functioning of these alveolar macrophage compromise the host so that it is unable to defend itself against a wide variety of opportunistic pathogens? This is the ultimate test! Although each of these effects on the macrophage may be statistically significant, what is the biological meaning of these responses?

A number of different experimental approaches, using intact animals, have been employed in an effort to determine the degree of efficiency of the pulmonary defense system in the pollutant-exposed, compromised host (*61,63,134,141,218–223*).

A most successful model is to introduce viable, opportunistic pathogens within the respiratory system. The animals whose host defenses are functioning normally are capable of returning the lung to its sterile condition within a few hours. However, if the defenses have been significantly altered to a degree that permits the viable organism to establish itself and to multiply, this ultimately will result in a noticeable increase in pulmonary infection and possibly death. In order for this infectious model system to be most effective and valid as a predictive tool, it must be (1) sensitive to subtle effects caused by low concentrations of the test material; (2) able to reflect the total summation of all of the deleterious changes in the most pulmonary defense system; (3) valid across species; (4) useful for the testing of a wide range of both gaseous and particulate pollutants; and (5) supported by mechanistic experimental information (*134,224*). When these criteria are met, the extrapolation to a similar effect, i.e., increased pulmonary infections occurring in man, becomes reasonable providing the microorganism present is capable of multiplying and invading that host and that the actual dose of the inhaled pollutant reaching these defenses is sufficient to cause an adverse effect.

Evidence exists that a number of trace metals which alter either biochemical, physiological, morphological or functional mechanisms of the macrophage also cause a significant increase in susceptibility to infection. Examples of some of the particles that have been shown to cause an enhancement of bacterial pulmonary infection include: Ni^{2+}, Cd^{2+}, Zn^{2+}, Pb^{2+}, H_2SO_4 and coal fly ash (*35,37,55, 62,74,117,132,219,222,225*). It is important to note that in many of these studies, dose-response data indicated that an effect could be produced with concentrations similar to those seen in the working environ-

ment. Neither iron oxide nor pure carbon dust produced any significant effect even at high concentrations.

Inhalation of a number of noxious gases and vapors has also been shown to increase pulmonary infections in this system. These gaseous pollutants also affect the functioning of the macrophage. Individual gases such as O_3 and NO_2 as well as mixtures of NO_2 and O_3, H_2SO_4 and O_3, irradiated auto exhaust and diesel exhausts have all caused significant enhancement of bacterial pneumonia in test animals (*114,141,219,220,226–228*).

Decreased resistance to influenza viral pneumonia, as a consequence of exposure to either NO_2, SO_2 or H_2SO_4 has been reported (*132,229,230*). Other studies have produced conflicting evidence (*231, 232*). A number of factors may be responsible for this variability such as: differences in specific virus used; concentration of viruses deposited in the lung; species and strains of animals tested; sequence of exposure and the time interval between exposure to the pollutant and the laboratory-induced challenge to this infectious agent.

Several epidemiological studies have emphasized the relationship between increased concentrations of pollutants and increased incidence of acute respiratory symptoms. Increased incidence of acute respiratory illness in humans have been associated with exposure to SO_2, suspended nitrates and sulfates, NO_2, cigarette smoke, total suspended particles and cooking with gas stoves (NO_2) (*163,167,233–241*).

Recently it has been shown that healthy subjects and asymptomatic asthmatics were unresponsive, i.e., no change in airway function, after exposure to sodium nitrate (*235*). Even after challenge with a parasympathomimetic agent (carbachol), this pollutant (NO_3^-) caused no significant airway response. However, subjects with acute respiratory disease (influenza) develop airway hyperreactivity after inhalation of the pollutant. The response was a specific effect of the nitrate since no significant constriction developed with exposure to sodium chloride. This study suggests that the presence of an acute viral respiratory illness may be a major determinant defining host response to specific pollutants. Such data indicate the need for further studies using individuals with respiratory disease as well as normal subjects.

Based upon these types of epidemiologic and acute animal studies it is reasonable to conclude that there is a synergistic relationship between exposure to pollutant chemicals and impaired resistance to pulmonary disease. It thus becomes apparent that the response to an environmental challenge is not a simple phenomena, but instead is a reflection of several different influences acting on the host simultaneously. The success or failure of the host to defend itself depends largely on the functioning of its interlocking pulmonary defenses.

This report has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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